Bufadienolides and Other Constituents of *Urginea sanguinea*

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Abstract: **Fresh bulbs of *Urginea sanguinea* yielded stigmasterol, phloroglucinol, phloroglucinol 1-β-D-glucopyranoside (phlorin), scillaren A, a novel compound 5α-4,5-dihydroscillaren A (1), salicylic acid, and 3-hydroxy-4-methylbenzoic acid. The latter two showed weak antibacterial activity. The compounds were identified using spectroscopic techniques such as 1D and 2D NMR, EI-MS and FAB-MS.**

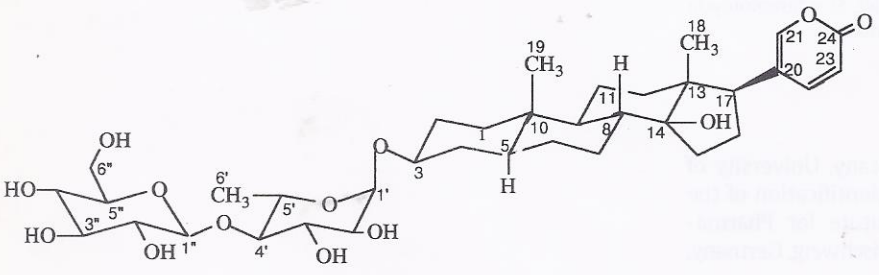
Urginea sanguinea (Schinz) Jessop (*Drimia sanguinea* Schinz) Liliaceae (Hyacynthaceae) is common in Southern Africa, with preference for hilly areas (1). The bulb is red and bleeds when cut, hence the name *sanguinea*. This species had not been investigated phytochemically until recently (2) but *U. sanguinea* is known to be among the most important poisonous plants hazardous to livestock in South Africa (2). After ingestion of the leaves, cattle, sheep, and goats show symptoms typical of cardiac glycoside poisoning (3). Tswana herbalists and traditional doctors use *U. sanguinea* to treat venereal diseases (1).

The ^{1}H- and ^{13}C-NMR (Table **1**) spectra of **1** indicated a glucose, a rhamnose and a steroid nucleus. Several structural features were evident from the 400 MHz ^{1}H-NMR spectrum of **1**. Clearly defined signals at δ = 6.27 (d, J = 9.7 Hz, H-23), 7.43 (d, J = 2.1 Hz, H-21) and 7.98 (dd, J = 2.1 and 9.7 Hz, H-22) are characteristic of protons constituting the δ-pyrone ring (4). The nature and stereochemistry of glycosyl moieties viz α-L-rhamnosyl and β-D-glucosyl were determined by anomeric proton resonances, respectively, at δ = 4.83 (d, J = 1.4 Hz, H-1′ and 4.57 (d, J = 7.8 Hz, H-1″). The steroid nucleus showed two methyls, eight methines, nine methylenes, and five quaternary carbons. The structure determination and assignments for **1** were achieved using COSY, HC-direct, HMBC, HOHAHA techniques and these data were consistent with the structure of **1** being a bufadienolide, a class of compounds which is very common in the genus *Urginea* (5–7). Most proton signals from rhamnose and glucose units were all superimposed and overlapped greatly. To resolve this signals HOHAHA was used to locate the individual spin systems of these sugar residues.

Table 1 ^{13}C-NMR (δ_C, 100 MHz) data for scillaren A and **1**.

Carbon atom	Scillaren A*	**1****
1	31.9	38.5
2	26.8	30.5[b]
3	72.6	77.5
4	120.7	35.3
5	146.4	45.7
6	34.9	30.6[b]
7	28.3[a]	28.9
8	41.5	43.0
9	49.5	51.4
10	37.0	37.1
11	20.9	22.7
12	39.7	41.9
13	47.9	49.2
14	83.0	86.1
15	28.5[a]	33.3
16	28.4[a]	30.2[b]
17	49.9	52.4
18	16.6	17.5
19	18.3	12.9
20	122.4	125.2
21	149.3	150.6
22	147.4	149.5
23	114.2	115.5
24	161.4	165.0
1′	99.0	99.6
2′	70.4	72.8
3′	70.5	72.6
4′	82.2	83.8
5′	67.0	68.6
6′	17.7	18.3
1″	105.5	105.9
2″	74.5	76.2
3″	76.6	78.2
4″	70.0	71.6
5″	77.0	78.3
6″	61.2	62.9

* DMSO-d_6.
** CD_3OD.
[a–b] assignments in each column interchangeable.



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Complete assignment of the sugar protons was done using HC-direct (Table **2**), HMBC (Table **2**), and HOHAHA (Table **3**). The order in which the two sugars were attached to the aglycone was determined by HMBC which showed a correlation between H-3 (δ_H = 3.52–3.59) and the rhamnose anomeric carbon, C-1′ (δ_C = 99.6, d). This connectivity was further confirmed by an HMBC between the rhamnose anomeric proton (δ_H = 4.83) and the aglycone C-3 (δ_C = 77.5, d). The rhamnose was therefore attached directly to the C-3 of the aglycone moiety. The sequence of sugar residues was also determined by HMBC which showed a cross peak between the rhamnose H4′ proton (δ_H = 3.58–3.63) and the glucose anomeric carbon (δ_C = 105.9, d). This was further confirmed by another HMBC cross peak between the glucose anomeric proton (δ_H = 4.57) and the rhamnose C-4′ (83.3, d) carbon.

Table 2 HC-direct and HMBC correlations for **1**.

Protons	Carbon		
^1H	^1J	^2J	^3J
7.98	149.5 d		150.6 d, 165.0
7.43	150.6 d	125.2 s	52.4 d, 149.5 d, 165.0 s
6.29	115.5 d	165.0 s	125.2 s
2.54 t	52.4 d	30.2 t, 49.2 s, 125.2 s	41.9 t, 86.1 s, 149.5 s, 150.6 d
0.71 (Me)	17.5 q	49.2 s	41.9 t, 52.4 d, 86.1 s
1.64–1.70	30.2 t	52.4 d	49.2 s, 86.1 s
1.67–1.75; 1.32–1.36	22.7 t	41.9 t	43.0 d
1.59	43.0 d	86.1 s	49.2 t
0.82 (Me)	12.7 q	37.1 s	38.5 t, 45.7 d, 51.4 d
0.96–1.00	51.4 d		
1.06–1.13	45.7 d		12.7 q
3.52–3.59 m	77.2 d		99.6 d*
4.83 d	99.6 d	72.8 d	68.8 d, 72.6 d, 77.5 d
3.75	72.8 d	72.6 d	83.8 d
3.57–3.59	83.8 d	68.8d	18.3 q, 72.8 d, 105.9 d*
1.31 d	18.3 q	68.8 d	83.8 d
4.57 d	105.9 d		83.3 d*
3.21–3.23	76.2 d	78.2 d, 105.9 d	
3.27–3.28	78.2 d		78.3 d
3.34–3.35	71.6 d		76.2 d
3.30–3.31	78.3 d		78.2 d
3.68–3.70	62.9 t	78.3 d	
3.83–3.86	62.9 t	78.3 d	

* Key HMBC correlations.

Table 3 HOHAHA correlations for **1** at 30 ms spin lock time.

^1H proton	HOHAHA correlation (30 ms)
H1′	H2′
H2′	H3′, H4′, H5′
H3′	H2′, H4′, H5′, Me6′
H4′	H3′, H4′, H5′, Me6′
H5′	H3′, H4′, Me6′
Me6′	H3′, H4′, H5′
H1″	H2″, H3″, H4″
H2″	H1″, H3″, H4″
H3″	H1″, H2″, H4″, H5″, H6″A, H6″B
H4″	H1″, H2″, H3″, H5″, H6″A, H6″B
H5″	H1″, H2″, H3″, H4″, H6″A, H6″B
H6″A, H6″B	H2″, H3″, H4″, H5″
H17	H16α, H16β, H15β
H16α	H17, H16β, H15β
H16β	H17, H16α, H15β
H15α	H15β
H15β	H17, H16α, H16β, H15α
H21	H23, H22
H22	H23, H21
H23	H22, H21

The assignment of the aglycone moiety was done using HMBC, HC-direct and HOHAHA. The carbons which could not be assigned using the above methods were assigned using literature assignments for related compounds (5). The FAB-MS confirmed the molecular weight and the sugar sequence. The spectrum gave a peak at *m/z* 533 indicating the loss of a glucose residue and another peak at *m/z* 387 showing the loss of a glucose followed by the loss of a rhamnose moiety. The above information led to the determination of the structure of **1** as 14-hydroxybufa-20,22-dienolide-3-*O*-α-L-rhamnopyranosyl-4′-β-D-glucopyranoside (5α-4,5-dihydroscillaren A) which has not been previously reported.

The antibacterial activity of the crude water extract, the *n*-BuOH fraction and pure compounds are shown in Table **4**. Salicylic acid is a well known antibacterial and preservative. However, it only showed activity at a loading of 100 μg/disc. Phenolic compounds like phloroglucinol which are expected to show antibacterial activity are not active at this loading but phloroglucinol derivatives show impressive activity against the same microorganisms (8, 9). Other phenolic compounds which show good activity are gallic acid (10) and caffeic acid esters (11). The activities of the isolated compounds compare very well with those of two antibacterial flavonoid glycoside derivatives tiliroside and platanoside (12). The toxic components (bufadienolides) make the plant unsafe to use it as a medicinal plant.

Table 4 Antibacterial activity of *Urginea sanguinea* and its constituents compared to chloramphenicol.

Organism	Compound	Loading (μg/disc)	Zone of inhibition (mm)
S. aureus	chloramphenicol	10	18
	chloramphenicol	25	20
	chloramphenicol	50	25
	crude water extr*	500	9
	n-butanol fr**	100	10
	salicylic acid	100	7.5
	3h-4-m-b	100	7
B. subtilis	chloramphenicol	10	20
	chloramphenicol	25	25
	chloramphenicol	50	31
	crude water extr*	500	8
	n-butanol fr**	100	9
	salicylic acid	100	8
	3h-4-m-b***	100	≤ 7

* Extr = extract.
** fr = fraction.
*** 3h-4-m-b = 3-hydroxy-4-methylbenzoic acid.

Materials and Methods

M.p.'s uncorrected: Kofler Hot Stage. *NMR*: on Bruker AMX 400; ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz), in either DMSO-d_6 or CD_3OD. ^{1}H- and ^{13}C-NMR chemical shifts were referenced to the residual solvent signals. *MS*: HREI AEI-MS 902 (70 eV) or a VG ZAB-E FAB MS. *IR*: Mattson Genesis Series FT-IR or a Perkin-Elmer 781, KBr discs. *UV spectra*: in CH_3OH on CE 505 UV Spectrophotometer. *Specific rotations* $[\alpha]_D$: Polatronic-D.

U. sanguinea bulbs were collected in Kgale, 10 km south of Gaborone, in May 1993, identified by Drs. L. Turton and B. Hargreaves and a voucher specimen (USRM 001) was placed at the National Herbarium, Gaborone, Botswana. *Extraction*: bulbs (3 kg) were chopped in pieces, extracted with cold water, concentrated, then freeze-dried to give 200 g residue. The crude water extract (195 g) was dissolved in distilled water (400 ml) and partitioned successively between 4 × 300 ml aliquots each of *n*-hexane, EtOAc, and *n*-BuOH. The extracts were evaporated *in vacuo* to yield 0.5, 1.5, and 120 g crude fractions, respectively. The residual water extract was freeze-dried to give 74 g of solid. The fractions were each tested (in triplicate) for antibacterial activity at a loading of 500 and 100 μg/disc using the disc diffusion assay (13, 14). *Separation*: The *n*-butanol extract (100 g) was added to a polyamide (300 g) column and eluted in a step-wise manner with toluene, toluene/EtOH (1 : 1), EtOH, EtOH/MeOH (1 : 1), MeOH, MeOH/$(CH_3)_2CO$ (1 : 1) and $(CH_3)_2CO$. Twenty-two fractions (150 ml each) were collected and the first seven fractions gave fatty acids and stigmasterol (50 mg).

TLC analyses of fractions 8–13 showed two major compounds and two minor ones. These fractions were purified on 100 g polyamide column and eluted with EtOH, EtOH/MeOH (1 : 1), MeOH, MeOH/H_2O (1 : 1) and H_2O in that order. The two major compounds were found in the first four (100 ml each). HPLC of these using a semi-preparative column (RP-18, MeOH/H_2O 68 : 32, flow rate 10 ml/min) led to isolation of scillaren A (65 mg), [t_R = 10 min] and 5α-4,5-dihydroscillaren A (26 mg), [t_R = 11.2 min].

Fractions 14–17 were put on Sephadex LH-20 (10 g, eluted with MeOH/$CHCl_3$, 1 : 1) and fractions containing the three minor components were further purified on HPLC (conditions same as above) to yield 3-hydroxy-4-methylbenzoic acid (6 mg) [t_R = 12.1 min] and salicylic acid (5 mg) [t_R = 13.4 min]. Fractions 18–22 showed two major spots which gave a bright red colour on spraying with 1% vanillin in sulphuric acid and heating. These were put on Sephadex G-10 (MeOH/H_2O 1 : 1). Ten fractions (50 ml each) were collected. Fractions 3–6 to yielded pure phlorin (78 mg) and fractions 8–10 gave phloroglucinol (24 mg).

5α-4,5-Dihydroscillaren A (**1**): Needles (from methanol), m.p. 192–194 °C, $[\alpha]_D$: −44.4 (*c* 0.12, MeOH) and formula $C_{36}H_{54}O_{13}$. UV λ_{max} (MeOH) nm: 298. IR υ_{max} (KBr) cm^{-1}: 3394, 2931, 1712, 1636, 1541, 1450, 1064. ^{1}H-NMR (400 MHz in CD_3OD): δ_H (*J* values in brackets) = 0.71 3H s, 0.82 3H s, 1.31 d (6.2), 2.05–2.08 m, 2.16–2.18 m, 2.54 t, 3.21–3.23 m, 3.27–3.28 m, 3.30–3.31 m, 3.34–3.35 m, 3.37 d, 3.57–3.59 m, 3.61 m, 3.68–3.70 dd (12.0, 4.2), 3.75 s, 3.83–3.86 dd (12.0, 1.6), 4.57 d (7.8), 4.83 d (1.4), 6.27 (9.7), 7.43 (2.1), 7.98 (2.1, 9.7). ^{13}C-NMR (see Table **1**). FAB-MS: 717 (M + Na)$^+$, 695 (M + H)$^+$, 533 (M + H − 162 (glucosyl))$^+$, 387 (M + H − 162 − 146 (rhamnosyl))$^+$, 369 (aglycone + H − H_2O)$^+$, 351.

Scillaren A: Needles (from methanol), m.p. 270 °C dec., $[\alpha]_D$: −68.6 (*c* 0.82, MeOH). UV, IR, NMR and MS data agreed well with literature data (5, 6).

Stigmasterol: (^{1}H-NMR, MS, R_f, colour reaction with vanillin sulphuric acid) matched those of an authentic sample of stigmasterol.

Phloroglucinol: Brown amorphous powder, m.p. 117 °C. UV, IR, NMR and MS consistent with literature data (15).

Phlorin: Brown amorphous powder, m.p. 231–233 °C, $[\alpha]_D$: −71.0 (*c* 0.10, MeOH). UV, IR, NMR and MS data showed good agreement with literature data (15).

Salicylic acid: Needles, m.p. 158–160 °C. Spectral data were consistent with literature data (16).

3-Hydroxy-4-methylbenzoic acid: Needles, m.p. 215 °C (dec). UV λ_{max} (MeOH): 250, 272, 278 sh. IR υ_{max} (KBr): 3422, 2920, 1706, 1630, 1529, 1449, 1236, 1064. ^{1}H-NMR (400 MHz in CD_3OD): δ_H = 2.19 3H s, 7.09 d (8.2), 7.19 dd (2.2, 8.2), 7.49 d (2 Hz). ^{13}C-NMR (100 MHz in CD_3SOCD_3): δ_C 16.2 q, 115.4 d, 120.3 d, 129.5 s, 129.6 s, 130.3 d, 155.4 s, 167.8 s.

Microorganisms and antibacterial assay: The test organisms consisted of *Staphylococcus aureus* (NCTC 6751), *Bacillus subtilis* (NCTC 8326), *Escherichia coli* (NCTC 9001) and *Pseudomonas aeruginosa* (NCTC 6750). The agar disc diffusion assay (13, 14) was used to screen the crude water extract, the fractions and purified compounds. Paper discs (6 mm diameter) containing 500 and 100 μg of sample were placed on agar plates inoculated with test organisms. Experiments were carried out in triplicate and discs of standard antibiotic (chloramphenicol) were used as a positive control. The presence of a clear zone of inhibition after 18 hours was taken as a positive result.

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